

Please insert the following paragraphs on page 88, line 5 of the specification.

--BIOLOGICAL EXAMPLES

In Vitro raf Kinase Assay:

In an *in vitro* kinase assay, raf was incubated with MEK in 20 mM Tris-HCl, pH 8.2 containing 2 mM 2-mercaptoethanol and 100 mM NaCl. This protein solution (20 μ L) was mixed with water (5 μ L) or with compounds diluted with distilled water from 10 mM stock solutions of compounds dissolved in DMSO. The kinase reaction was initiated by adding 25 μ L [λ - 33 P]ATP (1000-3000 dpm/pmol) in 80 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1.6 mM DTT, 16 mM $MgCl_2$. The reaction mixtures were incubated at 32 °C, usually for 22 min. Incorporation of ^{33}P into protein was assayed by harvesting the reaction onto phosphocellulose mats, washing away free counts with a 1% phosphoric acid solution and quantitating phosphorylation by liquid scintillation counting. For high throughput screening, 10 μ M ATP and 0.4 μ M MEK was used. In some experiments, the kinase reaction was stopped by adding an equal amount of Laemmli sample buffer. Samples were boiled 3 min and the proteins resolved by electrophoresis on 7.5% Laemmli gels. Gels were fixed, dried and exposed to an imaging plate (Fuji). Phosphorylation was analyzed using a Fujix Bio-Imaging Analyzer System.

All compounds exemplified displayed IC_{50} s of between 1 nM and 10 μ M.

Cellular Assay:

For *in vitro* growth assay, human tumor cell lines, including but not limited to HCT116 and DLD-1, containing mutated K-ras genes were used in standard proliferation assays for anchorage dependent growth on plastic or anchorage independent growth in soft agar. Human tumor cell lines were obtained from ATCC (Rockville MD) and maintained in RPMI with 10% heat inactivated fetal bovine serum and 200 mM glutamine. Cell culture media and additives were obtained from Gibco/BRL (Gaithersburg, MD) except for fetal bovine serum (JRH Biosciences, Lenexa, KS). In a standard proliferation assay for anchorage dependent growth, 3×10^3 cells were seeded into 96-well tissue culture plates and allowed to attach overnight at 37 °C in a 5% CO_2 incubator. Compounds were titrated in

media in dilution series and added to 96-well cell cultures. Cells were allowed to grow 5 days typically with a feeding of fresh compound containing media on day three. Proliferation was monitored by measuring metabolic activity with standard XTT colorimetric assay (Boehringer Mannheim) measured by standard ELISA plate reader at OD 490/560, or by measuring ^3H -thymidine incorporation into DNA following an 8 h culture with $1\ \mu\text{Ci}$ ^3H -thymidine, harvesting the cells onto glass fiber mats using a cell harvester and measuring ^3H -thymidine incorporation by liquid scintillant counting.

For anchorage independent cell growth, cells were plated at 1×10^3 to 3×10^3 in 0.4% Seaplaque agarose in RPMI complete media, overlaying a bottom layer containing only 0.64% agar in RPMI complete media in 24-well tissue culture plates. Complete media plus dilution series of compounds were added to wells and incubated at $37\ ^\circ\text{C}$ in a 5% CO_2 incubator for 10-14 days with repeated feedings of fresh media containing compound at 3-4 day intervals. Colony formation was monitored and total cell mass, average colony size and number of colonies were quantitated using image capture technology and image analysis software (Image Pro Plus, media Cybernetics).

In Vivo Assay:

An *in vivo* assay of the inhibitory effect of the compounds on tumors (e.g., solid cancers) mediated by raf kinase can be performed as follows:

CDI nu/nu mice (6-8 weeks old) are injected subcutaneously into the flank at 1×10^6 cells with human colon adenocarcinoma cell line. The mice are dosed i.p., i.v. or p.o. at 10, 30, 100, or 300 mg/Kg beginning on approximately day 10, when tumor size is between 50-100 mg. Animals are dosed for 14 consecutive days once a day; tumor size was monitored with calipers twice a week.

The inhibitory effect of the compounds on raf kinase and therefore on tumors (e.g., solid cancers) mediated by raf kinase can further be demonstrated *in vivo* according to the technique of Monia et al. (*Nat. Med.* **1996**, *2*, 668-75).--